OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT INITIATION

Date: September 6, 1978

Project Title: Non-Heme Iron Oxygenase Catalysis

Project No: G-33-H02

Project Director: Dr. Sheldon W. May

Sponsor: DHEW/PHS/NIH - National Institute of General Medical Sciences

Agreement Period: From 9/1/78 Until 8/31/79 (02 Year)

Type Agreement: Grant No. 5 R01 GM23474-02

Amount: $57,003 New PHS Funds (G-33-H02)
7,011 GIT Contribution (G-33-330)
$64,014 Total

Reports Required: Annual Progress Reports with Continuation Applications
Terminal Progress Report upon Grant expiration

Sponsor Contact Person (s):

Technical Matters

John C. Dalton, Ph.D
(Dr. Eugene J. Oliver)
Dep. Associate Director for
Program Activities
National Institute of General Medical
Sciences
Bethesda, MD 20014

Phone: 301-496-7518

Contractual Matters
(thru OCA)

Evelyn W. Carlin
Grants Management Officer
Office of Assoc. Director for
Program Activities
National Institute of General
Medical Sciences
Bethesda, MD 20014

NOTE: FOLLOW-ON PROJECT TO G-33-H01.

Defense Priority Rating: None

Assigned to: Chemistry (School/Laboratory)

COPIES TO:

- Project Director
- Division Chief (EES)
- School/Laboratory Director
- Dean/Director--EES
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- EES Reports & Procedures
- Project File (OCA)
- Project Code (GTRI)
- Other

CA-3 (3/76)

GEORGIA INSTITUTE OF TECHNOLOGY OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION SHEET

Date 8/2/83

Project Title: Non-heme Iron Oxygenase Catalysis

Project No: G-33-H02

Project Director: Sheldon W. May

Sponsor: DHEW, Public Health Services

Effective Termination Date: 8/31/79

Clearance of Accounting Charges: ----

Grant/Contract Closeout Actions Remaining:

None

- ☐ Final Invoice and Closing Documents
- ☐ Final Fiscal Report
- ☐ Final Report of Inventions
- ☐ Govt. Property Inventory & Related Certificate
- ☐ Classified Material Certificate
- ☐ Other _____

NOTE: "Section IV - Summary Progress Report" submitted as part of application for G-33-H03 constitutes annual report required for G-33-H02 here. Two xc's of that Section obtained and enclosed for Library Archieves.

Assigned to: Chemistry (School/Laboratory)

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Other _____

SECTION IV

APPLICANT: REPEAT GRANT NUMBER SHOWN ON PAGE 1 →	GRANT NUMBER
SECTION IV—SUMMARY PROGRESS REPORT	GM-23474-03

PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial)	PERIOD COVERED BY THIS REPORT	
May, Sheldon W.	FROM	THROUGH
NAME OF ORGANIZATION: Georgia Institute of Technology	9/1/78	8/31/79

TITLE (Repeat title shown in Item 1 on first page)
Non-heme Iron Oxygenase Catalysis G-33-402/yr 1(

1. List publications: (a) published and not previously reported; (b) in press. Provide five reprints if not previously submitted.
2. List all additions and deletions in professional personnel and any changes in effort.
3. Progress Report. (See Instructions)

1a."Preparation and Properties of Cobalt (II) Rubredoxin," S. W. May and J. Y. Kuo, Biochemistry, 17, 3333 (1978).

"A Resonance Raman Study of Substrate and Inhibitors Binding to Protocatechuate-3,4-dioxygenase," R. H. Felton, L. D. Cheung, R. S. Phillips and S. W. May, Biochem. Biophys. Research Comm., 85, 844 (1978).

"Enzymatic Epoxidation Reactions," S. W. May, Enzyme Microb. Technol., 1, 15 (1979).

"A Kinetic and Raman Investigation of Substrate and Inhibitor Binding to Protocatechuate-3,4-dioxygenase," R. S. Phillips, S. W. May, L. D. Cheung and R. H. Felton, Fed. Proceed., 38, 2603 (1979).

"Separation Techniques Based on Biological Specificity," S. W. May and L. M. Landgraff, Recent Developments Separation Science, 5, 227 (1979).

"Affinity Chromatography," S. W. May, Techniques of Chemistry, 12, 257 (1978).

1b. "Protocatechuate-3,4-dioxygenase: Implications of Ionization Effects on Binding and Dissociation of Halohydroxybenzoates and on Catalytic Turnover," S. W. May and R.S. Phillips, Biochemistry,

"Enzymatic Oxygenation of Hydrocarbons," R. S. Phillips and S. W. May, preprints Petroleum Division American Chemical Society, in press.(1979).

2. The personnel associated with this project will remain virtually the same as last year. Mrs. Laura Landgraff has completed her Master's degree and will no longer be associated with this project. Dr. Robert Phillips has completed his Ph.D. working with protocatechuate-3,-4-dioxygenase, and will continue to work part time on this project.

No changes in effort are planned.

(see continuation page next)

3. PROGRESS REPORT

Objectives. Non-heme iron containing oxygenases have been shown to be involved in a large number of metabolic processes in both mamalian and bacterial systems. The goal of our research program is to analyze the involvement of non-heme iron in the catalytic pathway of a selected oxygenase. Particular attention is to be paid to the possibility of changes in coordination geometry during catalysis, to the relationship between the substrate binding site and the metal ion, to the factors which influence substrate binding and to the possible role of protein functional groups in catalysis. Of particular significance, we wish to evaluate fluorine-containing substrate analogs as potential specific and effective oxygenase inhibitors which should be highly useful active-site probes. Hopefully, the results obtained will alow for the design of similar compounds potentially useful as specific oxygenase inhibitors for use in the clinical treatment of disease.

Isolation of Protocatechuate-3,4-Dioxygenase and Syntheses of Active-Site Directed Substrate Analogs. These operations are now carried out routinely using the procedures we developed during the first year of the project as described in last year's report.

Resonance Raman Spectroscopic Studies. Our goals for this year were to complete the resonance Raman spectroscopic studies on both PCD and its E·S and E·I complexes in collaboration with Dr. R. H. Felton of this department. We have previously demonstrated that 3-halo-4-hydroxybenzoates such as 3-FHB and 3-ClHB are potent inhibitors and active-site titrants which exhibit simple kinetic and spectral properties, and our comparative studies with 3- and 4-substituted hydroxybenzoates established the critical role of the 4-hydroxl substituents in binding. Insight into the molecular details of metal participation in these binding interactions has now been provided by our resonance Raman studies on resting PCD and both the E·S and E·I complexes. We have demonstrated that the iron atom in PCD is tyrosine ligated, an observation now confirmed in two other laboratories and consistent with CD studies and we have been able to estimate that, in fact, two tyrosines are involved in iron coordination. In the E·I complexes with the 3-halohydroxybenzoates, our data establish that inhibitor interacts with the metal atom via Fe-O ligation without displacing either tyrosine, thus clarifying the critical need for a p-hydroxyl substituent for potent binding. In the case of substrate, the stable E·S species observed anaerobically in our Raman studies exhibits iron chelation by the *o*-dihydroxy grouping, again with neither tyrosine ligand being displaced. In all probability, the carboxylate functionality of both substrate and inhibitors ionically interacts with a cationic group, likely an arginine [by analogy with *p*-hydroxybenzoate hydroxylase] or possibly a lysine. Thus,resonance Raman has provided an unambiguous molecular -level picture of the active-site region of PCD and the changes which occur upon substrate and inhibitor binding. During the coming year, we wish to extend these Raman studies to the mechanistically significant ternary $E \cdot S \cdot O_2$ complex which has been implicated as an obligatory intermediate in catalysis.

Our Raman data have also suggested the intriguing possibility that there is a sulfhydryl ligand to the iron atom, which is displaced upon inhibitor binding. The existence of such a sulfhydryl ligand is reminiscent of the situation in P-450, but here the iron atom is in an entirely difference coordination environment. We plan to follow up on this suggestion by carrying out further spectroscopic studies as well as specific chemical modification work coupled with reconstitution experiments. In this regard, the availibility in our hands of iron, cobalt-substituted and specifically thiol-modified rubredoxin is of critical importance [see Biochemistry, 17, 3333 (1978)].

3. Progress Report (continued)

Kinetic Studies of Inhibitor and Substrate Binding and Catalytic Turnover. In a series of experiments which beautifully complement the Raman, we have completed both steady state and rapid reaction kinetic studies on inhibitor and substrate binding and on catalytic turnover. We have previously shown that the E·I complexes of all inhibitors containing 4-OH substitutents exhibit virtually identical spectral features. Thus, at first glance it seems natural to conclude that the increase inhibitory potency caused by 3-halo substitution is attributable to the greatly increased acidity of the *p*-hydroxyl. Our kinetic data establish that prior ionization is not, in fact, responsible for this increase binding potency, and furthermore, that the ionization effects on substrate and inhibitor association rate constants are very similar. It seems reasonable to assume that the monodentate Fe-O ligation which we see in the Raman of E·I also occurs for substrate on the pathway toward chelation, the stable state actually seen in the Raman studies on the E·S complex. Thus, we conclude that the active site of PCD is set up to provide for facile removal of the proton from even a non-acidic *p*-hydroxyl such as those found in all known PCD substrates. This could be accomplished by the presence of a base (e.g. a histidine),which picks up the proton as the hydroxyl nears the coordination sphere of the iron, effectively lowering its pK_a. This picture, explains why PCD readily accomodates either the protonated or anionic forms of the halohydroxybenzoates, and in order to accomdate the kinetic and spectral data, we conclude that the ionization state of this base has a negligible effect on either the spectral or kinetic properties of the E·I complex. A strikingly similar conclusion has very recently been reached regarding p-hydroxybenzoate hydroxylase. Our data on inhibitor binding clearly reveals a high sensitivity to the steric requirements of the substitutents at position 3 and a linear free energy plot shows a very good correlation with steric substituent constants.

To further elucidate the action of PCD with inhibitor, dissociation rates were measured via stopped-flow double displacement experiments on several classes of inhibitors. The results are not discussed here in detail, but fully corraborate the picture of inhibitor binding at the PCD active site just described.

We have also completed a detailed study of ionization effects on the breakdown of $E{\cdot}S{\cdot}O_2$, and have demonstrated that a simple ionization of pk 7 facilitates this process. In a manuscript which has been submitted for publication we discuss this finding in terms of currently thinking about the mechanism of action of dixoygenases such as PCD.

In summary, we have used kinetic and spectroscopic techniques to amplify on the molecular-level picture of binding at the active site of PCD provided by our Raman work. In addition, the kinetic results have provided insights into the detailed mechanism of oxygen insertion catalyzed by this non-heme iron containing dioxygenase. During the coming year, our goals are to focus further on the coordination environment of the metal with particular attention to the possibility of a sulfhydryl ligand and to utilize both spectroscopic and kinetic techniques to elucidate the structure of the catalytically competent $E{\cdot}S{\cdot}O_2$ complex, thus providing molecular-level detail about the mechanism of non-heme iron dioxygenase catalysis.

Significance. It is our contention that information of the type which we are attempting to obtain with PCD is highly significant. As such information becomes available for non-heme oxygenases, it will serve as the basis for the design of oxygenase inhibitors and/or activators for possible *in vivo* applications similar to those currently being pursued with other types of enzymes (e.g. proteases). We feel that our work with

3. Progress Report (continued)

the fluoro-substituted substrate analogs is especially significant in this regard since it establishes the potential of such compounds as highly potent oxygenase inhibitors. Second generation effectors can now be designed, and we anticipate incorporating reactive chemical functionalities into such substances so as to render them irreversible or "suicide" effectors for various oxygenases. Even if the particular compounds we are now examing never prove directly useful in clinical applications, they will be exceedingly useful tools for the study of the role of oxygenases in biological processes. Thus, if such compounds only establish a general approach for affecting disease-related oxygnease enzymes, they will represent a major step forward towards the eventual design of clinically useful compounds.

The undersigned agrees to accept responsiblity for the scientific and technical conduct of the project and for the provision of the required progress reports if a grant is awarded as a result of this application.

6/11/79
Date

Sheldon W. May
Principal Investigator